Title

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Journal

Malaria journal, 1(1)

ISSN

1475-2875

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Publication Date

2002-08-01

DOI

10.1186/1475-2875-1-10

Peer reviewed

Malaria Journal

Research

Behavioural determinants of gene flow in malaria vector populations: *Anopheles gambiae* males select large females as mates

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Published: 14 August 2002

Malaria Journal 2002, **1**:10

Received: 7 June 2002
Accepted: 14 August 2002

This article is available from: http://www.malariajournal.com/content/1/1/10

Abstract

Background: *Plasmodium*-refractory mosquitoes are being rapidly developed for malaria control but will only succeed if they can successfully compete for mates when released into the wild. Pre-copulatory behavioural traits maintain genetic population structure in wild mosquito populations and mating barriers have foiled previous attempts to control malaria vectors through sterile male release.

Methods: Varying numbers of virgin male and female *Anopheles gambiae* Giles, from two strains of different innate sizes, were allowed to mate under standardized conditions in laboratory cages, following which, the insemination status, oviposition success and egg batch size of each female was assessed. The influence of male and female numbers, strain combination and female size were determined using logistic regression, correlation analysis and a simple mechanistic model of male competition for females.

Results: Male *An. gambiae* select females on the basis of size because of much greater fecundity among large females. Even under conditions where large numbers of males must compete for a smaller number of females, the largest females are more likely to become inseminated, to successfully oviposit and to produce large egg batches.

Conclusions: Sexual selection, on the basis of size, could either promote or limit the spread of malaria-refractory genes into wild populations and needs to be considered in the continued development and eventual release of transgenic vectors. Fundamental studies of behavioural ecology in malaria vectors such as *An. gambiae* can have important implications for malaria control and should be prioritised for more extensive investigation in the future.

Background

Malaria remains one of world's most burdensome and intractable diseases [1–3]. As the Roll Back Malaria campaign proceeds to promote the effective implementation of existing control methods, one of the most rapidly developing new strategies is the possibility of rendering wild vector populations less susceptible to infection by releasing genetically-modified mosquitoes [4–7]. Transposable genetic elements may allow relatively small releases of such transgenic laboratory-reared mosquitoes to drive *Plasmodium*-refractory genes to fixation in wild vector populations, even if these genes confer substantially reduced fitness [8,9]. However, the fixation of malaria-refractory genes will ultimately depend upon the ability of the laboratory-reared transgenics to survive and mate with wild vectors following their release [9]. Recent studies have also shown that the fitness costs of insecticide resistance genes in mosquitoes can include reduced mating competitiveness among males [10]. Pre-copulatory behavioural barriers are therefore not only important because of their influence upon the genetic structure of wild vector populations [11] but also our future ability to prevent these insects from spreading malaria [9]. Recently, the utility of transgenic mosquitoes as a malaria control tool has been seriously questioned and the ecology of targeted vector species has been identified as a poorly understood topic which needs to be investigated in much more detail before any such releases can be envisaged in the future [5,7,8].

Unfortunately most investigations of mating behaviour in mosquitoes have concentrated on species that are active during the day [12] whereas the important vectors of malaria are predominantly crepuscular or nocturnal species such as *An. gambiae* Giles [13]. We have therefore quantified determinants of mating and oviposition success in *An.gambiae*, one of the world's most effective vectors of malaria [14,15] and found that males compete aggressively for selected females based on the size of the latter.

Materials and methods

Experimental conditions

The bulk of this work was carried out using the Ifakara strain of *An. gambiae*, originally colonized from wild caught gravid females in the Kilombero valley, Tanzania. For the cross-strain mating experiments, the Mbita strain, similarly colonized in Western Kenya were used and both strains were reared under semi-natural conditions in separate screen-walled greenhouses exposed to ambient airflow and meteorological conditions on the shores of Lake Victoria, in Western Kenya [16–18]. All experiments were conducted in 30 cm cubic netting cages within additional screen-walled greenhouses where females were placed in paper cups within cages containing males 1 hr before release. The mosquitoes were released and allowed to interact within the cage between 1600–0800 h. For experiments to identify determinants of insemination success and fecundity, all females were offered bloodmeals on three consecutive days and then individually transferred to separate glass vials with a paper strip to rest on, 6% glucose and an oviposition pad. These mosquitoes were then allowed five days to oviposit following which the insemination status of non-ovipositing females determined by dissection and examination of the spermathecae

Mechanistic modeling of mating success as a function of competition

Firstly, it was assumed that all females are receptive to insemination but that the ability of males to inseminate females depends on whether other competing males are present. We define these abilities in terms of the rate at which single and additional competing males inseminate individual females, denotes I_1 and I_c. We assume that a single male inseminates a given, limited proportion of females and that insemination of remaining females by additional competing males progresses at a rate proportional to the number of additional males (M). Assuming that, for all experiments, the amount of time allowed for mating was constant (one night), and applying a standard first order decay function, the proportion remaining uninseminated (U) is an exponential function of the proportion remaining uninseminated by a single male:

$$U = (1 - I_1) \exp - (I_c (M-1)) \qquad \text{Equation 1}$$

The proportion of females inseminated in each cage as a function of male density was fitted to this equation separately for each number of females, pooling all 3 replicates to give 18 observations. This nonlinear regression was applied using the NLIN procedure of SAS 8.0, fitted by maximum likelihood weighted according to the binomial variance and bounding U to values between 0 and 1 as well as I_1 and I_c to positive values. In order to express the level of competition under any given conditions, we define the competition index (C) as the rate at which individual males alone in a cage succeed in inseminating females divided by the rate at which those in competition with others do so:

$$C = I_1 / I_c \qquad \text{Equation 2}$$

Results

Using the Ifakara strain of *An.gambiae*, we found that peaks of insemination activity at dusk and dawn were associated with an elliptical flight pattern, typical of swarming dipterans [12], in which a number of male and female mosquitoes form a cluster on one side of the cage. Males routinely jostled with each other in flight and often dis-

Table 1: Final fitted logistic regression model accounting for variations in probability of female insemination as a function of male and female numbers per cage and replicate. Deviance and Pearsons χ^2 goodness of fit statistics were 3.35 and 3.31 per degree of freedom, respectively, with 49 degrees of freedom and P < 0.0001 for both.

Factor	$\beta \pm$ SEM	P
Intercept	-0.599 ± 0.070	<0.0001
Reciprocal of females per cage	6.12 ± 1.79	0.0006
Males per cage	0.00586 ± 0.00054	<0.0001

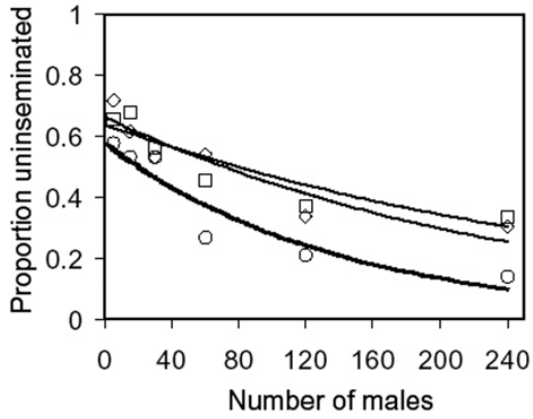

Figure 1
The insemination success of females as a function of the numbers of males and female per cage. The proportion of uninseminated females is plotted as lines representing of the fitted competition model (See materials and methods) at each level of female numbers per cage (See table 2). The mean of observations at each number of males per cage (n = 3) are represented by circles, diamonds and squares for 10, 30 and 100 females per cage, respectively.

placed others from their resting positions on the netting. Male flight was very rapid with several individuals flying through the same loops, pursuing each other while facing in the same direction. It was during these interactions that males most frequently succeeded in grasping and copulating with females. The females flew much less than the males and although transient flights over short distances occasionally occurred, they were most commonly observed when grasped by males. Consistent with previous reports [19,20], one single male was found to inseminate up to 10 females per night (Mean $\pm$ SEM = 8.3 ± 1.0, n = 4 experiments) when placed in cage with 30 of them. However, the addition of 1, 2 or 3 males (that is doubling, tripling or quadrupling the male numbers in a cage) did not increase the proportion of the 30 females that became inseminated. This suggests that males compete with each other for the same limited proportion of females rather than distributing their attention to inseminate as many females as possible.

In order to study the level of competition amongst males in more detail and to determine whether the number of females present also influences mating success, we measured insemination success of differing numbers of females with a wider range of male numbers. Virgin female batches of 10, 30 or 100 were combined with 5, 15, 30, 60, 120 or 240 virgin males under standardized conditions overnight and the insemination status of every female was inspected the following morning. Stepwise logistic regression analysis revealed that both female and male numbers in each cage significantly influenced insemination probability of females (Table 1). Note however, that although significant, the influence of both male and female numbers are surprisingly small (Figure 1). If, for example, females simply competed with each other for a limiting number of inseminations made available by the males, one would expect increasing the number of females in the cage from 10 to 30, and then 100, to reduce the proportion inseminated by three- and ten-fold, respectively. Clearly this is not the case (Figure 1), indicating that although adding 30 or more females to a cage may slightly reduce the insemination success, this is not due to direct competition between females and the availability of insemination opportunities to them is not limiting. In order to determine whether the observed insemination patterns could be adequately described by such simple competition mechanisms, the same data were fitted to a simple mechanistic model, in which we combined common kinetic approaches with our empirical observations (See materials and methods).

This model simply assumed that all females are receptive to insemination but that the ability of males to inseminate females depends on whether other competing males are present or not. The model was derived and fitted to the data in Figure 1 in order to test whether the observed patterns of insemination could be explained by such simple competition mechanisms and to estimate and compare the success rates of competing and non-competing males (see Materials and Methods). The three different levels of females per cage were fitted separately and found to be consistent with the proposed model (Figure 1, Table 2). The close fit of the model to the observations indicates that most, if not all, females are available and receptive to males. Estimates of the insemination rates for non-competing (I_1) and competing (I_c) males (Table 2) were consistent with those estimated by logistic regression for the intercept and the dependence of insemination success on

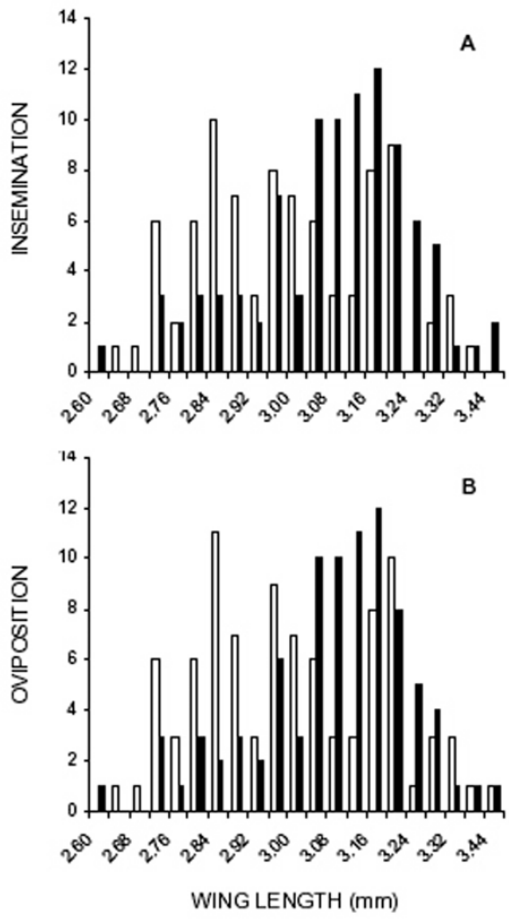

Figure 2
Insemination and oviposition success of females as a function of size. Empty and solid bars represent the number of females that respectively failed and succeeded in (**A**) becoming inseminated and (**B**) ovipositing in the pooled groups of different mosquito strains and competition levels carried out in experiment 3 (See Table 3).

male numbers per cage, respectively (Table 1). Interestingly, the number of females per cage, over the range tested, did not consistently influence the proportion that became inseminated by one lone male (Table 2). This means that, as more females are made available to the males, they inseminate the same proportion but correspondingly larger numbers of females. Thus the availability of sperm does not appear to be the factor that limits insemination success under these conditions but rather the selection of the most attractive females. At low female numbers, the additional proportion of females inseminated by each competing male was twice that of the two higher levels of female numbers (Table 2). Consequently, the level of competition amongst males increased ($P < 0.01$ by Spearmans ρ test) with the numbers of females per cage (Table 2), suggesting that crowding may allow increased selectivity of males for females. Greater size has been shown to be associated with both prolonged swarming and successful insemination within swarms, by wild *An. freeborni* males [21] and increased fecundity in females of the *An.gambiae* complex [22,23] so we investigated this relationship in more detail with three successively more detailed experiments.

An initial experiment confirmed previous reports [22,23] that oviposition likelihood depended on the size of females and this observation was consistently repeated in the two subsequent experiments that examined this relationship in more detail (Table 3, Figure 2). This first experiment also showed that increased competition actually reduced the likelihood of oviposition, despite the increased insemination probability expected (Table 3). Thus the second experiment examined the likelihood of both insemination and subsequent oviposition by individual females, under conditions of high and low male competition (Table 3). Both insemination and oviposition were much more likely for larger females (Table 3, Figure 2). Interestingly, of those females inseminated, the larger ones were no more likely to oviposit than the smaller ones. Also, increased male competition levels increased insemination probability but reduced oviposition probability in females in general and in those that were successfully inseminated, indicating that males became less discriminate when competing with each other, resulting in choice of females less likely to reproduce.

A third experiment was then conducted to determine whether size could mediate barriers to gene flow between different strains of *An. gambiae* (Table 3). The Mbita strain, colonized from the immediate environs of the study site in Western Kenya are consistently larger than the Tanzanian Ifakara strain used for all the previous experiments. Even though both strains are routinely reared under identical conditions in large-scale insectaries at Mbita Point Research and Training Centre [16–18], Mbita females are consistently larger ($P < 0.001$) with a mean wing length of 3.102 ± 0.013 mm compared with 2.958 ± 0.020 mm for Ifakara females. We therefore repeated experiment 2 but with all four combinations of strains and sexes. Furthermore, the eggs laid by each female were collected, counted and reared to pupae to assess the viability of all resulting offspring. The two strains were found to interbreed readily and no influence of strain combination on insemination or overall oviposition probability nor on oviposition probability or total number of eggs laid by inseminated females could be detected (Table 3). All strain combinations appeared viable and approximately 60% of all egg

Table 2: The ability of males to inseminate females as a function of competition and female density. Estimates for the proportion of females inseminated by the addition of one male only (I_1) and the rate at which they are inseminated by additional competing males (I_c) were estimated by fitting a simple mechanistic model and the competition index (C) calculated as their quotient (See materials and methods).

Females per cage	I_1	I_c	P*	C
10	0.422 ± 0.053	0.0073 ± 0.0016	0.992	58 ± 15
30	0.338 ± 0.042	0.0040 ± 0.0009	0.932	84 ± 21
100	0.362 ± 0.045	0.0031 ± 0.0009	0.336	116 ± 37

* Pearsons goodness of fit statistic

Table 3: Dependence of female insemination probability and fecundity upon size, male competition level and strain.*

Experiment	N	Outcome	Parameter	β ± SEM	P
Ifakara strain only					
1 & 2	91	Oviposition†	Intercept	-16.5± 5.6	<0.001
			Size	5.4 ± 1.8	0.001
			Competition	-1.44 ± 0.59	0.009
2	46	Insemination†	Intercept	-38.1 ± 10.7	<0.001
			Size	13.1 ± 3.7	<0.001
			Competition	1.91 ± 1.0	0.034
		Oviposition after insemination†	Intercept	0.15 ± 0.56	0.782
			Competition	-1.95 ± 0.95	0.024
All four male-female combinations of Ifakara and Mbita strains					
3	180	Insemination†	Intercept	-13.0 ± 3.4	<0.001
			Competition	2.37 ± 0.38	<0.001
			Size	3.98 ± 1.12	<0.001
	180	Oviposition†	Intercept	-10.2 ± 3.1	0.001
			Competition	1.83 ± 0.34	<0.001
			Size	3.06 ± 1.01	0.003
	94	Oviposition after insemination†	Intercept	2.52 ± 0.39	<0.001
	87	Egg batch size of ovipositors‡	Intercept	-1.16 ± 0.47	0.017
			Size	0.94 ± 0.15	<0.001

* Size was measured as wing length (mm) and male competition was either low or high (5 versus 50 males per cage, respectively, with 30 females). † Logistic regression model fitted by forward conditional stepwise selection ‡ Generalized linear model fitted manually including only terms found to be significant upon testing all candidates. The size of egg batches was log-normally distributed and correspondingly transformed so parameter estimates refer to their influence upon Log_{10} (egg batch size +1).

batches were successfully reared to pupae. However, size and competition level were significant determinants of both insemination and oviposition success in all crosses (Table 3, Figure 2). The association between insemination and wing size was confirmed using correlation analysis of the same data using Spearman's non parametric ρ test (Correlation coefficient = 0.254, P = 0.01) and a parametric partial correlation test, controlling for strain combination (Correlation coefficient = 0.249, P = 0.01). Oviposition likelihood following insemination was independent of maternal strain, paternal strain, competition and female size. However, female size, and no other factor, was found to be a determinant of the size of egg batches in ovipositing females, indicating that, regardless of strain, males choose the largest females, presumably because they lay more eggs (Table 3, Figure 3).

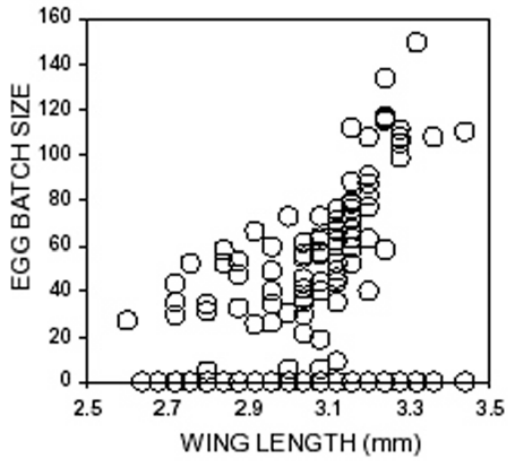

Figure 3
Egg batch size as a function of size in the pooled groups of different mosquito strains and competition levels carried out in experiment 3 (See Table 3). Each circle represents the egg batch of one mosquito.

Discussion

In contrast with *An. freeborni*[21], recent field studies of *An.gambiae* have shown that large males are no more likely to fall out of mating swarms in copula than are small males [24]. Here, however, we have shown that the size of female *An.gambiae* has major impact on not only their fecundity [22,23] but also their chance of being chosen as mates by the males who compete intensely for the privilege. Under these laboratory conditions, female size is the major factor influencing mate choice by males within and between strains as well as the size of subsequent egg batches. The behaviour by colony-adapted mosquitoes within the confines of relatively small cages may not necessarily reflect equivalent processes in wild field populations and further investigation in larger semi-field systems [16–18,25] and natural populations are clearly required. Although the studies we have described here do illustrate the kind of ethological determinants of gene flow that may exist in nature, further investigation in the field is essential because the process of adapting wild mosquitoes to colony rearing and maintenance inevitably selects for behaviour that is compatible with successful mating under artificially high densities within the confines of small cages. Nevertheless, our observations are fully consistent with current insect sexual selection theory, which indicates that the choosiness of males has evolved in response to strong correlation between the selected trait and fecundity of females [26]. Such closely associated sexual and reproductive fitness traits, as well as natural selection based upon them, are therefore likely to be important in wild field populations of *An.gambiae* from which these captive strains were derived and may have important practical implications for malaria control in the future.

Conclusions

Here we have shown that the size of female *An. gambiae* may be a critically important determinant of their reproductive fitness in terms of their ability to compete for mates and their ability to lay large egg batches. In practical terms this may have important implications for the success of malaria control measures such as the release of genetically modified mosquitoes that are refractory to malaria because such strategies are inherently dependent upon the reproductive fitness of the released mosquitoes [8,9]. For example, rearing conditions for breeding batches for release will need to be carefully considered because overcrowding or poor nutrition of larvae result in smaller, reproductively disadvantaged adults [22,27]. Similarly, size heterogeneities arising from variability in larval habitat quality [28,29] may affect gene flow patterns in the field and need to be considered when driving exogenous genes into field populations which are spatially and temporally structured [9]. Beyond their direct implications, our observations also illustrate how fundamental studies of behavioural ecology in mosquitoes can have important implications for malaria control and should be prioritised for more extensive investigation in the future [7,11].

Competing interests

None declared.

Acknowledgements

We thank V. Robert and P. Billingsley for insightful comments and support from NIH through International Centre for Infectious Disease Research (U19-AI45511), Actions in Building Capacity (D43-TW01142) and Kenya-Mali Collaborative Exchange (D43 TW00920) awards.

We also thank S. Bopp for assistance with preliminary experiments and P. Ongele for technical assistance in the insectary.

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